Gross clinical signs and haematological changes associated with artificial infection of *Edwardsiella tarda* in Koi Carp

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Abstract

The occurrence of disease is a significant setback for successful aquafarming. One of the common fish bacterial disease syndromes, Edwardsiellosis is caused by *Edwardsiella tarda*, a gram–negative, rod shaped bacterium associated with several diseases of marine and fresh water fish. In this study, an attempt was made to observe and analyze the onset of clinical symptoms and certain haematological parameters in Koi Carp, *Cyprinus carpio* L., following artificial infection with *Edwardsiella tarda.*

The disease progress was observed and the clinical symptoms were monitored over a period of 15 days following infection. Fish were sampled at three day intervals to analyse the haematological parameters: total erythrocyte counts (RBC), total leucocyte counts (WBC), haemoglobin content and differential leucocyte count. Clinical symptoms observed included: erratic swimming behaviour, loss of appetite, haemorrhages, dropsy and exophthalmia. There was a significant decrease in the total RBC and haemoglobin levels by the 3rd and 6th day post infection, and an increase thereafter. WBC counts were higher in all infected groups in comparison to the control group. A significant increase in the number of neutrophils was found in the infected group upto the 9th day and a decrease thereafter. The lymphocyte number was significantly less upto the 12th day while the monocyte counts were significantly higher upto the 12th day post infection.

The results showed that the bacterium, *E. tarda*, is pathogenic to Koi Carp. The hematological changes and clinical signs in infected fish reported in this paper will be helpful in the identification and the control of this infection.

Keywords: Koi Carp, *Edwardsiella tarda,* artificial infection, clinical symptoms, haematology

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Introduction

Koi carps are the ornamental variety of the common carp (*Cyprinus carpio* L.). Although this fish is hardy, they are susceptible to disease under stress conditions. Bacteria are the most common pathogens of cultured warm water fish, and can cause major losses to the fresh water aquaculture industry in India and elsewhere. They are also the most prevalent cause of morbidity and mortality among wild populations of fish. Among the most damaging bacterial infections, motile Aeromonads and *Edwardsiella* spp. are the most significant. *Edwardsiella tarda* is a commonly found pathogen causing Edwardsiellosis (emphysematous putrefactive disease) leading to mass mortality in various populations and age groups of fish. Fish species commonly affected by this condition include carp, tilapia, eel, catfish, mullet, salmon, trout and flounder (Mohanty and Sahoo, 2007).

Edwardsiella tarda is a gram negative, motile short rod shaped bacterium (1 µm in diameter and 2-3 µm long), which infects a wide range of hosts including both fresh water and marine fish (Thune *et al*., 1993). Although the pathogen does not appear to be invasive, under favourable environmental conditions, the organism is capable of causing heavy mortality. This organism is important not only because of its pathogenecity to fish, but also for its role in gastroenteritis, wound infection, meningitis, and urinary tract infections in man. The actual role of the microorganisms may vary from that of a primary pathogen to that of an opportunistic invader rendering the host moribund by initiating a disease process.

A disease is expressed in terms of clinical signs and symptoms: gross and histopathological lesions, clinical manifestations provide the initial clue to the nature of the infection. In addition to the identification of the bacteria, understanding the hematological changes in infected fish will be helpful in the identification and subsequent control of the disease.

All aquarium fish including Koi carps are susceptible to this pathogen and there are no effective methods for treatment, control or prevention of the condition at present. A proper understanding of the disease condition, therefore, is the most important step in controlling the pathogen. In the present experiment, certain gross clinical signs and haematological changes were studied in Koi carp artificially infected with *E. tarda.*

Materials and Methods

Fish

Koi carps (*Cyprinus carpio* L.) were procured from ornamental fish farmers in Kurla, Mumbai. The mean length and weight of fish were 9.36 ± 0.074 cm and 11.73 ± 0.765 g, respectively. The experimental fish were acclimatized in 1000 L fiberglass tanks for one

week, before distributing them into rectangular plastic tanks with a capacity of 160 L. Half the water in the tanks was replaced daily which were provided with continuous aeration. A commercial feed (CP 4092, CP Aquaculture (India) Pvt. Ltd.) was fed at the rate of 5% of body weight.

Bacteria

Pure cultures of *Edwardsiella tarda* were inoculated in to Brain Heart Infusion (BHI) broth and were incubated for 24 hours at 30 °C. Loopfuls of the bacterial cultures from the broth were streaked on Rimmlar–Shott's medium (Himedia, India). The identification of the bacteria was confirmed by biochemical tests according to "Bergey's Manual of Determinative Bacteriology".

After incubation, the culture was transferred aseptically to sterilized 15 ml centrifuge tubes and centrifuged at 2200 g at 4 °C for 10 minutes in a cooling centrifuge (Biofuge Stratos-Heraeus). The supernatant was discarded and the pellet containing bacteria was re-suspended in a similar volume of phosphate buffered saline (PBS – pH 7.4); this process was repeated three times. Following the washing procedures, the number of bacteria in the suspension was quantified indirectly by using a spectrophotometer (Thermospectronic UV1) as well as directly by using a spread plate method in order to obtain an accurate estimate of the number of bacteria.

Experimental design

Six rectangular plastic tanks with lids (76.3 cm x 52.1 cm x 41.0 cm) of uniform size and 160 L capacity were used in the study. The tanks were initially disinfected with a solution of potassium permanganate (15 mg/l) for 24 hours and were cleaned thoroughly with water before stocking. Each tank (containing 145 L of chlorine free water) was stocked with twenty apparently healthy fish and conditioned for one week with sufficient aeration.

Fish were fed twice per day at 5% body weight at 7.00 am and 5.00 pm throughout the experimental period. Experimental tanks were cleaned manually by siphoning out half the water along with faecal matter and other debris daily and was replaced with freshwater. Water quality parameters were maintained below levels toxic to fish (temperature 25.6-28.5 °C; pH 7.6 - 8.4; dissolved oxygen (DO_2) 5.6-7.8 mg/l; unionized ammonia <0.025 ppm). Fish were observed daily for signs of stress.

Following conditioning, fish in three tanks were infected with *E. tarda* as described below; fish in the other 3 tanks were kept as controls. Fish were anaesthetized by immersing in a 4-5 ppm solution of clove oil and injected intra-peritonially with 100-150 µl of a suspension containing 5.75×10^7 CFU/ml of *E. tarda* culture. Control fish were injected with 100-150 µl of Phosphate Buffered Saline (PBS).

Sampling and haematology

All the inoculated fish were observed for clinical symptoms from 24 hours after infection. Three fish from each tank, treated and control, were sampled at intervals of three days, up to 15 days, for haematology. Blood was collected by puncturing the caudal vein of the fish and haematological measurements were made immediately.

Total erythrocyte and total leucocytes were measured using an improved Neubar ruling haemocytometer for counting (Hasser 1960). Haemoglobin concentrations were determined by the cyanmethhaemoglobin method using a diagnostic kit from Qualigen Diagnostics, India. Blood smears for differential counting of white cells were prepared from fresh blood, air dried and fixed in methanol and stained with Field's stain A and B (Himedia, India). All blood cells were identified as described by Hibia (1982) for the differential leucocyte count, a total of 100 leucocytes being observed.

Statistical analysis

Results are presented as mean ± standard error of means (SEM). The statistical significance of differences between the control and treatment means was assessed by a one-way analysis of variance followed by Duncan's new multiple range test. All statistical calculations were carried out using the SPSS software package (SPSS Inc., Chicago, IL, USA).

Results

Clinical symptoms and pathological changes

Within 24 hours of infection, the fish infected with *Edwardsiella tarda* became sluggish, showed rapid opercular movements and avoided feeding. Hemorrhages of the skin, wounds in belly area, abdominal distention and severe congestion in ventral area were evident between 24-48 hours post infection. With the progression of the disease, exophthalmia, scale erosion and extended wounds near the injected area were visible in 2-3 days. Those fish that became moribund showed severe congestion in the abdominal area and a protruded vent due to accumulation of fluid in peritoneal cavity (Fig. 1). All the other fish regained normal behaviour within 4-5 days. The wounds at the injection site persisted up to the 8^{th} day and healed naturally by the 15^{th} day.

Fig. 1. Severe congestion in abdominal area and protruded vent due to the accumulation of fluid in peritoneal cavity in moribund fish

Haematological changes

Total Erythrocyte (RBC) count

The infected group showed significantly ($p<0.05$) lower RBC counts up to the 12th day post infection in comparison to the control group. The RBC counts showed a reduction up to 6th day after infection and showed a gradual increase by the 9th and the 12th day and attained normal values by the 15th day post infection. Mean values for RBC counts are shown in Table 1.

Table 1. Mean RBC counts (±S.E) in Koi carp experimentally infected with *E. tarda*

Days post infection	Mean RBC counts (10^6 cells/mm^3) Control group	Mean RBC counts (10^6 cells/mm^3) Infected group
3	1.72^{d}±0.02	1.53^{bc}±0.02
6	1.73^{d}±0.12	1.41^{ab}±0.05
9	1.70^{cd}±0.02	1.48^{ab}±0.01
12	1.60^{cd}±0.02	1.47^{ab}±0.01
15	1.73^{d}±0.02	1.72^{d}±0.01

Different superscript values show significant differences ($P< 0.05$)

Total leucocyte (WBC) count

The treated group showed significantly ($p<0.05$) higher WBC counts in comparison to the control group. There was an increase in WBC counts until the 6th day post infection which then showed a decrease up to the 15th day, with the highest WBC counts being recorded on the 6th day post infection. The mean WBC counts are shown in Table 2.

Table 2. Mean WBC counts (±S.E) in Koi carp experimentally infected with *E. tarda*

Days post infection	Mean WBC counts (10^4 cells/mm^3) Control group	Mean WBC counts (10^4 cells/mm^3) Infected group
3	76.43^{a}±2.98	117.38^{c}± 1.69
6	74.03^{a}±3.47	142.65^{e}± 0.40
9	78.93^{a}±0.94	125.58^{d}± 1.98
12	75.73^{a}±2.60	113.23^{c}± 1.29
15	73.70^{a}±4.72	99.98^{b}± 0.89

Different superscript values show significant differences (P< 0.05)

Haemoglobin content

There was a significant decrease ($p<0.05$) in the total haemoglobin content in the infected group up to the 12th day post infection, with the lowest levels being recorded on the 6th day post infection. Thereafter, there was an increasing trend in hemoglobin values in the treated group. The mean Hb values are shown in Table 3.

Differential leucocyte count

There was a significant ($p<0.05$) increase in the number of neutrophils in the treated group. The highest number of neutrophils was recorded on 12th day post infection which then declined on the 15th day post infection. The lymphocyte numbers were significantly ($p<0.05$) less up to the 12th day in infected fish. Monocytes were significantly higher in the treated fish up to the 12th day of infection with the highest counts recorded on the 9th day post infection. The mean values for different leucocytes are shown in Table 4.

Table 3. Mean Hemoglobin levels of Koi carp infected with *E. tarda* (g/dl)

Days post infection	Control group	Infected group
3	7.79^{d}±0.03	6.31^{b}±0.10
6	7.77^{d}±0.01	5.21^{a}±0.01
9	7.94^{d}±0.07	6.15^{b}±0.02
12	7.86^{d}±0.10	6.99^{c}±0.05
15	7.81^{d}±0.04	7.76^{d}±0.00

Different superscript values show significant differences (P< 0.05)

Table 4. Mean white cell counts (±S.E) in Koi carp infected with *E. tarda*

Days post infection	Treatments	Lymphocytes	Neutrophils	Monocytes
3	Control	86.66^{d}±0.88	11.33^{a}±0.33	1.66^{a}±0.33
	Infected	73.75^{b}±0.85	21.25^{d}±0.62	5.00^{b}±0.40
6	Control	87.00^{d}±0.57	11.00^{a}±0.57	2.00^{a}±0.00
	Infected	70.25^{a}±0.85	23.50^{d}±0.64	6.00^{c}±0,07
9	Control	85.66^{d}±0.88	12.33^{ab}±0.33	1.66^{a}±0.33
	Infected	75.25^{b}±2.39	17.75^{c}±2.17	7.00^{d}±0.81
12	Control	87.00^{d}±0.57	11.33^{a}±0.33	1.66^{a}±0.33
	Infected	79.50^{c}±0.64	18.00^{c}±0.70	2.50^{a}±0.28
15	Control	87.00^{d}±0.57	12.00^{ab}±1.00	1.60^{a}±0.47
	Infected	84.00^{d}±0.40	15.00^{bc}±0.40	1.75^{a}±0.47

Means bearing different superscripts in the same column are significantly different ($p<0.05$)

Discussion

Edwardsiellosis is one of the most important bacterial diseases in fish. Fish affected by the disease show spiraling movements and die with the mouth agape and opercula flared, which may be associated with the development of anaemia leading to oxygen insufficiency. The fish reveal gross lesions on the skin, pale gills, tumefaction of the eyes, excessive mucus secretion, scale erosion and ulcers in a few cases. Swelling and bleeding of the anus leading to reddening is often noticed. In per acute cases congestion of the ventral part of the body is commonly seen (Meyer and Bullok 1973; Padros *et al.*, 2006). In mild infections, the only manifestation of the disease is the appearance of small cutaneous lesions (3-5 mm in diameter) on the postero-lateral parts of the body. As the disease progresses, abscesses develop within the muscle of the flank or caudal peduncle. In the acute stages these abscesses rapidly increase their size and develop in to large cavities filled with gas which are visible as convex, swollen areas. Loss of pigmentation over the lesions is common (Sahoo *et al*., 1998). If the lesions are incised, a foul odour is emitted. Necrotic tissue remnants may fill up to one third of the body cavity. As the infection progresses, affected fish lose control over the posterior half of the body (Meyer and Bullock, 1973). In our experiment, fish infected with *Edwardsiella tarda* were

sluggish, showed rapid opercular movements and avoided feeding, within 24 hours of infection. Haemorrhages of the skin, wounds in belly area, abdominal distension and severe congestion in the ventral area were prominent within 24-48 hours post infection. With the progression of the disease, exophthalmia, scale erosion, and extended wounds near the injected area were visible within 2 -3 days. In moribund fish, severe congestion in abdominal area and protruded vent due to accumulation of fluid in peritoneal cavity were observed. Similar observations were reported by Zhang *et al*., (2000) in their study. The wounds persisted up to the 8th day and naturally healed by the 15th day. Clinical signs including haemorrhages of the skin and the mouth with the presence of cutaneous ulcerations were observed by Benli and Yildiz (2004), in tilapia.

The data obtained in the present study highlighted the considerable haematological changes that take place in Koi carp following artificial infection with *E. tarda*. Infected fish showed significantly lower RBC counts up to the 12th day post infection in comparison to the control group. The RBC counts reduced up to the 6th day after infection and then gradually increased by the 9th and the 12th day and within the normal range on the 15th day post infection. A significant reduction of the erythrocyte count was also observed by Benli and Yildiz, (2004) for spontaneous infection of *E. tarda* in tilapia. There was a significant decrease in the total haemoglobin content in the infected group up to the 12th day post infection. Decreased haemoglobin counts were also detected by Benli and Yildiz, (2004). In general, the pattern of change was similar to the observation in bacterial infections (Brenden and Huizinga, 1986; Bruno and Munro, 1986; Yildiz, 1998). These results indicate that haematopoiesis may be severely affected in bacterial diseases (Barham *et al*., 1980).

The treatment group showed significantly higher WBC counts in comparison to the control group fish. There was an increase in WBC counts until the 6th day post infection which decreased thereafter up to the 15th day. Significant increase in the total leucocyte counts was detected by Benli and Yildiz (2004), in agreement with our findings. Increase in leucocyte counts during infection, associated with the organism's defense system against pathogens, is well known (Balfry *et al*., 1994; Yildiz, 1998; Caruso *et al*., 2002).

In the present study, there was a significant increase in the number of lymphocytes in all treatment groups. The highest number of neutrophil counts was recorded on the 12th day post infection and decreased thereafter. The lymphocyte numbers were significantly less up to the 12th day in the treatment groups. Monocyte counts were significantly higher up to the 12th day after infection. Highest monocyte counts were recorded on the 9th day post infection. A significant increase in neutrophils and monocytes in two fish species have been reported during the first 2-4 days post infection with *Renibacterium salmoninarum* (Bruno and Munro, 1986) which is in conformity with our results.

From the present study, it was evident that *E. tarda* is pathogenic to Koi carp and can produce clinical symptoms characterized by erratic swimming behaviour, anorexia, congested and oedematous vent, dropsy and exophthalmia. Observations made in this study indicated that most of the haematological parameters, namely, total RBC, and haemoglobin are depressed and WBC increases during the course of the disease, which lingers for 3 to 9 days. It was also observed that the fish can overcome the period of acute infection after about 10 days.

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